ORIGINAL CONTRIBUTION

# Fate of Immortalized Human Neuronal Progenitor Cells Transplanted in Rat Spinal Cord

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**Background:** Replacement of neurons and glia by transplantation has been proposed as a therapy for neurodegenerative diseases, including amyotrophic lateral sclerosis. This strategy requires using human motor neuronal progenitor cells or xenografts of animal cells, but there is little evidence that xenografted neuronal cells can survive in spinal cord despite immunosuppression.

**Objective:** To clarify the mechanisms responsible for the death of xenografted neurons in spinal cord.

**Methods:** Cells from an immortalized, neuronally committed, human embryonic spinal cord–derived cell line (HSP1) that expresses motor neuronal properties in vitro were transplanted into adult rat spinal cord. The rats were killed at intervals up to 8 weeks and serial sections through the graft sites were processed for immunofluorescence using primary antibodies against human nuclear and mitochondrial antigens, microtubule-associated protein 2, TUJ1, CD5, natural killer cells, and activated microglia-macrophages, caspase-3 and caspase-9.

**Results:** Grafted cells did not migrate and underwent partial differentiation along a neuronal pathway. They were rejected after 4 weeks despite cyclosporine immunosuppression. Cells died by apoptosis via the cytochrome *c*/caspase-9/caspase-3 pathway. The host response included natural killer cells and activated microglia-macrophages but few T cells.

**Conclusions:** Intraspinal neuronal xenotransplantation failed because of apoptotic cell death. Neither T cells nor the spinal cord environment, which favors gliogenesis, are likely to have been responsible, but natural killer cells may have been involved.

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NEURONAL AND GLIAL REplacement has been proposed as a treatment for neurodegenerative diseases, such as amyotrophic lateral sclerosis. This treatment requires either that human motor neuronal progenitors be used or that animal cells be used as xenografts. Evidence that xenotransplantation of neurons into the spinal cord can be successful is scant, even though immunological rejection is routinely suppressed with cyclosporine therapy. To elucidate why, an immortalized, neuronally committed, human embryonic spinal cord–derived cell line (HSP1) that expressed motor neuronal properties in vitro[1] was transplanted into rat spinal cord. The advantages of this approach include the following: (1) absence of the antibody-mediated hyperacute rejection phenomenon directed toward vascular endothelial glycoproteins in peripheral organ transplants[2]; (2) the reported relative absence of major histocompatibility complex (MHC) class II antigens in expanded neural precursor cells compared with primary cell suspensions[3]; (3) avoidance of ethical issues associated with harvesting human fetal or embryonic stem cells or solid spinal cord tissue; and (4) ultimately, avoidance of the intensified immunological rejection associated with xenotransplantation if human-derived cells can be developed for therapeutic use.

## METHODS

### CELL CULTURE

HSP1 cells (immortalized with the tetracycline/doxycycline–suppressible *v-myc* oncogene) were maintained in Dulbecco modified Eagle medium/F12 (DMEM/F12; Invitrogen Corporation, Carlsbad, Calif) supplemented with N2, HLA-B27, fibroblast growth factor 2 (50 ng/mL),

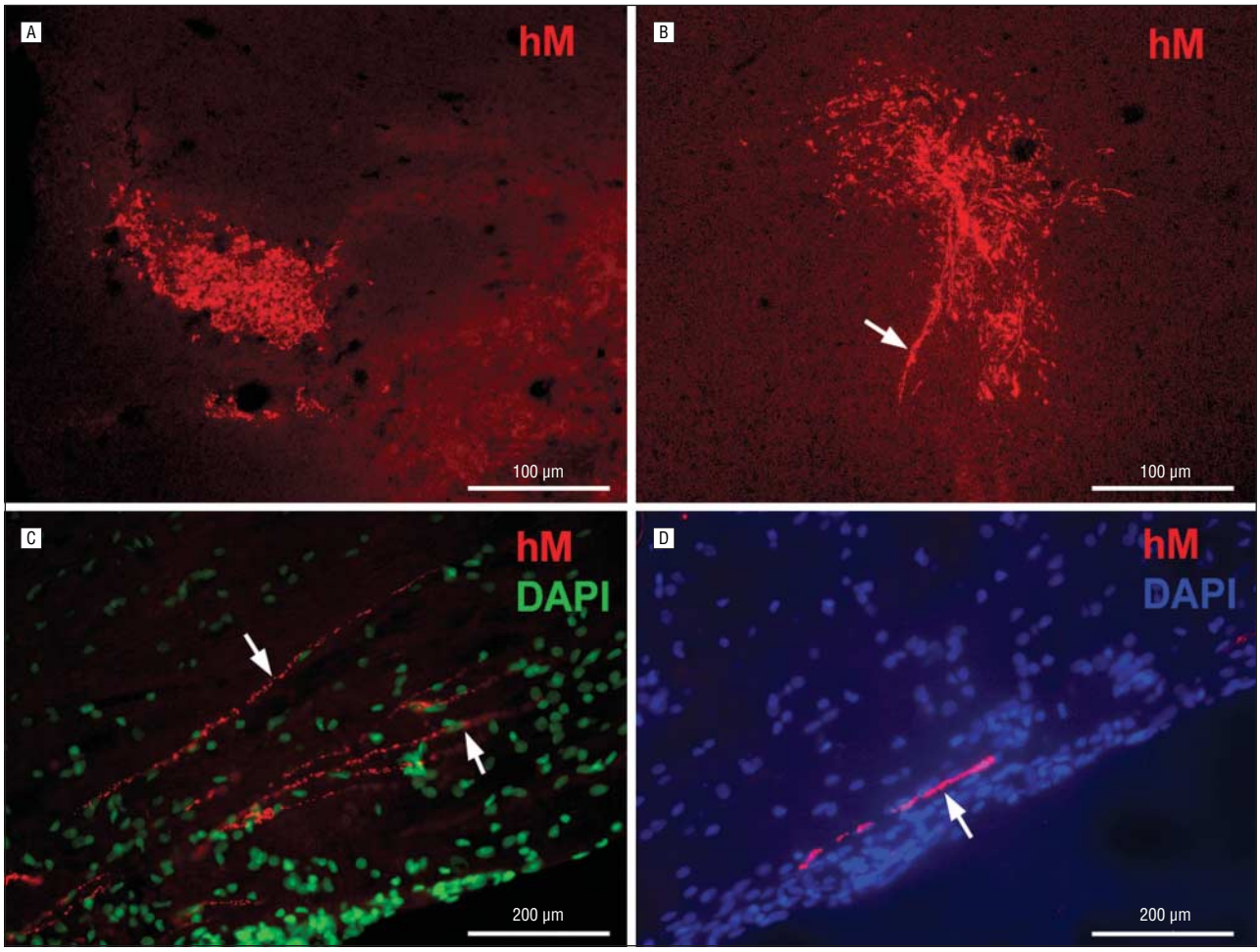


**Figure 1.** Identification of transplanted HSP1 cells with antihuman mitochondrial (hM) antigen. Cells were transplanted into animals treated with 1 mg/mL of doxycycline for the following times before being killed: A, 2 days; B, 1 week; C, 2 weeks; D, 6 weeks. Neuron processes contain mitochondria and are labeled by hM antigen (arrows). DAPI indicates 4′, 6′-diamidino-2-phenylindole. The color of the labels indicates location in the figure.

epidermal growth factor (50 ng/mL), insulin-like growth factor II (10 ng/mL), platelet-derived growth factor (10 ng/mL), and bovine serum albumin (2.5 mg/mL) in flasks coated with human plasma fibronectin (0.25 mg/mL). Cultures approaching confluence were passaged via trypsinization and split 1:5. On the day of surgery, 80% to 90% confluent cells were collected and resuspended at 50 000 cells/µL in DMEM/F12 and kept on ice. Cell viability, assessed by trypan blue exclusion at the end of the transplantation, averaged more than 90%.

## SURGERY AND TISSUE PROCESSING

All procedures were carried out in accord with a protocol approved by Drexel University College of Medicine's Institutional Animal Care and Use Committee, Philadelphia, Pa, and followed National Institutes of Health guidelines. Female Sprague-Dawley rats weighing 220 to 250 g were deeply anesthetized intraperitoneally with a combination of acepromazine maleate (0.7 mg/kg), ketamine (95 mg/kg), and xylazine (10 mg/kg), and the spinal cord exposed by a C4-5 laminectomy and dural incision. Fifty thousand HSP1 cells in 1 µL of DMEM/F12 were microinjected into the right side of the spinal cord through a glass micropipet (50- to 100-µm tip diameter) at 0.5 µL/min with a pump controller and the wound was closed. Hippocampal injections were carried out similarly with rats in a stereotaxic frame. Rats were immunosuppressed daily with cyclosporin A (15 mg/kg) subcutaneously, from 3 days prior to surgery until perfusion.

Animals survived between 2 days and 8 weeks. Some were given doxycycline in their drinking water (either 20 µg/mL or 1 mg/mL), changed daily for all or part of that time. They were killed with an overdose of pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, Ill) (80 mg/kg) and perfused transcardially with 0.9% isotonic sodium chloride solution followed by 4% paraformaldehyde in a 0.1M phosphate-buffered saline (PBS) solution. Brains and spinal cords were dissected the next morning and cryoprotected in 30% sucrose/0.1M PBS solution at 4°C for 2 to 4 days. Spinal cord segments of interest were embedded in OCT (Optimal Cutting Temperature) compound, frozen, and sectioned on a cryostat at 20 µm for spinal cord and 30 µm for brain tissue samples. Sections were mounted on gelatin/poly-L-lysine–coated glass slides and stored at –80°C.

## IMMUNOHISTOCHEMISTRY STUDIES

Serial sections were processed for immunofluorescence. Primary antibodies (sources available on request) included human nuclear (hN) antigen, human mitochondrial (hM) antigen, microtubule-associated protein 2 (MAP2), human-specific Ki-67, human nestin, TUJ1, CD5, natural killer (NK) cells, caspase-3, and caspase-9. Sections were washed 3 times, blocked in 10% goat or donkey serum containing 0.3% Triton X-100 for 1 hour at room temperature, and incubated in a PBS solution containing 0.3% Triton X-100, 2% goat serum, and pri-

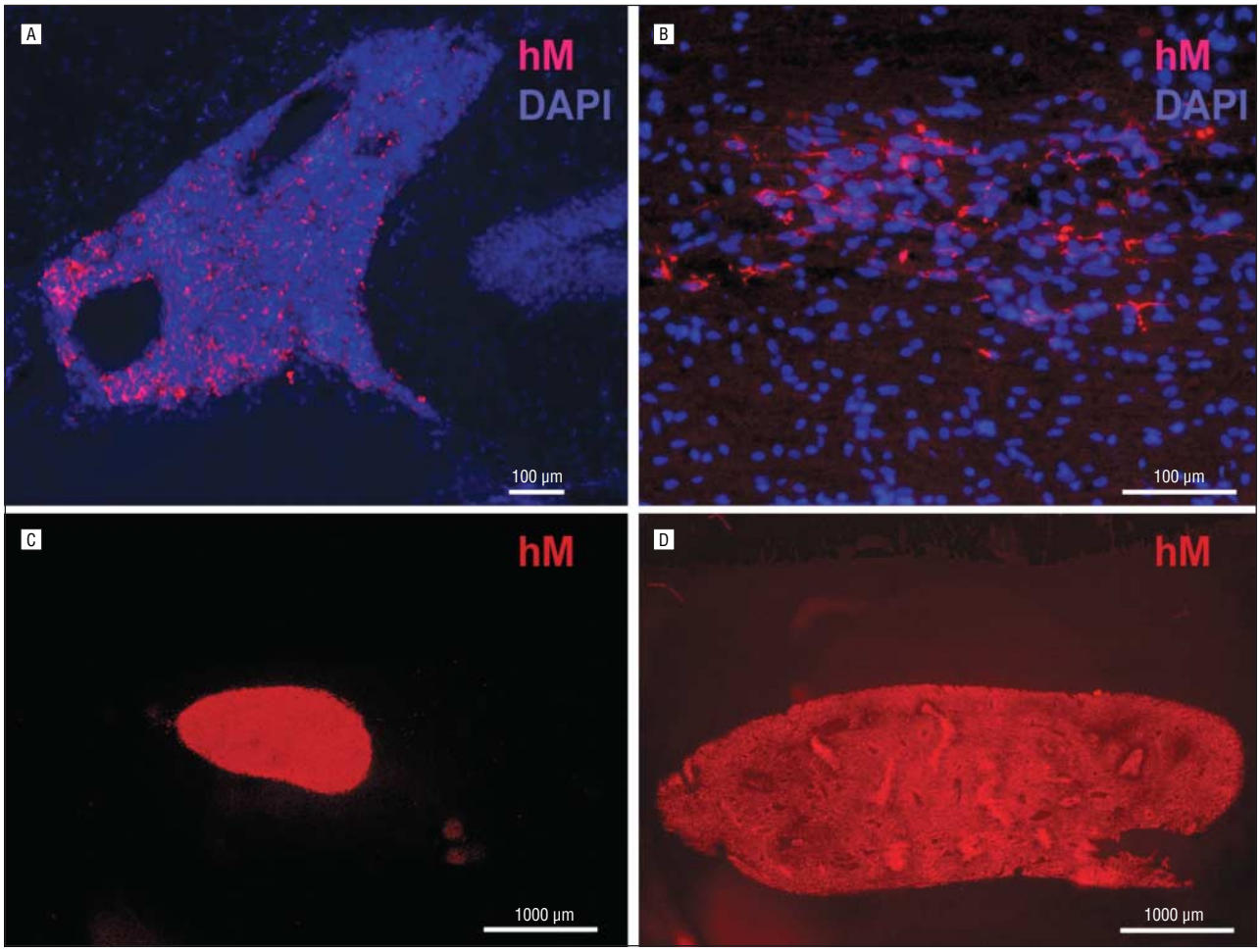


**Figure 2.** Cells grew better in the hippocampus than in the spinal cord and better in no doxycycline–treated animals than in low doxycycline–treated animals. HSP1 cells were transplanted into the hippocampus and spinal cord of animals given 20 µg/mL of doxycycline or no doxycycline, killed at 2 or 4 weeks after transection and stained for human mitochondrial (hM) antigen. A, Low-dose of doxycycline in the hippocampus at 2 weeks. DAPI indicates 4′,6′-diamidino-2-phenylindole. B, Low-dose doxycycline in the spinal cord at 2 weeks. C, No doxycycline in the spinal cord at 2 weeks. D, No doxycycline in the spinal cord at 4 weeks. The color of the labels indicates location in the figure.

mary antibodies overnight at 4°C. The second day sections were incubated in a PBS solution containing 0.3% Triton X-100, 2% goat serum, and specific rhodamine- or fluorescein isothiocyanate–conjugated secondary antibodies for 2 hours at room temperature. After 3 PBS solution rinses, cells were coverslipped with a mounting medium (Vectashield; Vector Laboratories Inc, Burlingame, Calif) with 4′,6′-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Ore). No staining was observed in controls, substituting respective normal serum samples for primary antibodies.

## RESULTS

### EXTENT OF SURVIVAL AND MIGRATION OF TRANSPLANTED HSP1 CELLS

Rats were given 1mg/mL of doxycycline for 2 days, 1 week, or 2 weeks after transplantation and killed at 2 days or 1 to 8 weeks after transplantation. Many HSP1 cells were present at 2 days and 1 week after transplantation (**Figure 1**A, B). Thereafter, the number of surviving HSP1 cells decreased dramatically. At 2 weeks, the cells became scarce, but of those that survived, many sent out long hM antigen–positive processes (Figure 1C). At 4 weeks, only a few hM antigen–positive fibers remained. At 6 weeks, rare HSP1 cells were found in only 1 of 4 animals (Figure 1D). No human cells were found at later times.

### ENVIRONMENTAL INFLUENCES ON TRANSPLANTATION SURVIVAL

To determine whether doxycycline toxicity or the spinal cord environment, which favors gliogenesis and does not normally support neuronal differentiation,[4] might be responsible for the disappearance of transplanted HSP1 cells, 50 000 cells were transplanted into the spinal cord and hippocampus of the same rats. These rats received 20 µg/mL of doxycycline for 1 or 2 weeks and were allowed to survive for 2, 3, 4, and 6 more weeks. In 4 of 4 two- and three-week animals, but only 1 of 5 longer-surviving animals, hM antigen– or hN antigen–positive cells were found in the hippocampus and spinal cord. Grafted cells did not emit long processes, were always found close to the injection site, and survived better in the hippocampus than in the spinal cord (**Figure 2**A, B; and **Table**).

**Table. Distribution of Surviving Transplanted HSP1 Cells With Low-Dose Doxycycline***

| Time After Transplantation, wk | No. of HSP1 Pos Rats/ Total Rats | HSP1 Pos Thickness in Brain, µm | HSP1 Pos Thickness In Spinal Cord, µm |
|---|---|---|---|
| 2 | 2/2 | 840 | 420 |
| 3 | 2/2 | 1155 | 280 |
| 4-6 | 1/5 | 1260 | 420 |

*Abbreviation: POS, positive.

Serial sections were examined for human nuclear antigen– or human mitochondrial antigen–labeled cells. The distribution of cells was estimated by calculating the total thickness of tissue in which labeled cells were found.

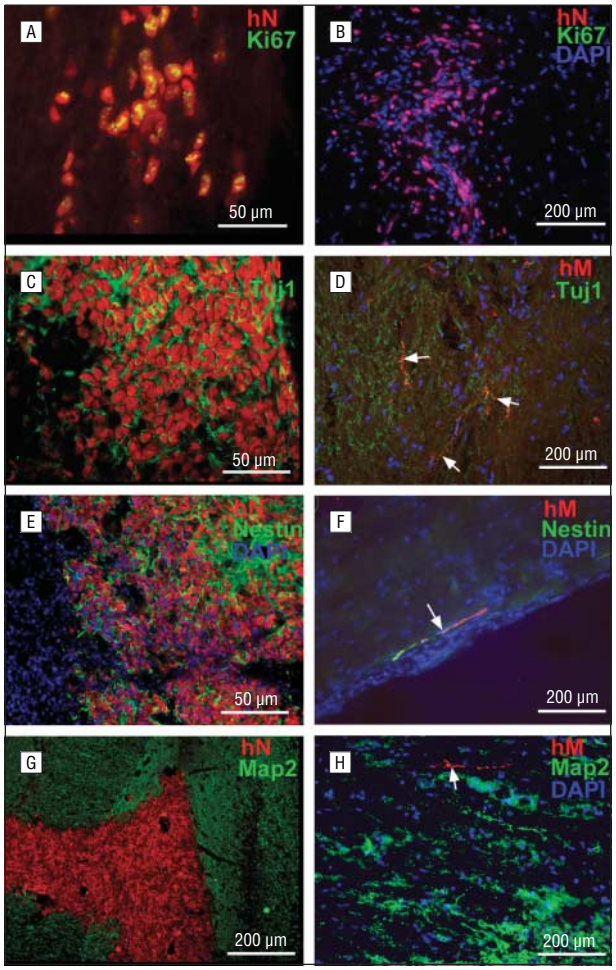


**Figure 3.** Cells showed partial differentiation only at high-dose doxycycline but survived better at low-dose doxycycline. HSP1 cells were transplanted into the spinal cord and animals given low-dose (left column) or high-dose (right column) doxycycline. Animals survived 1 to 3 weeks. Histological sections are double labeled for human antigens and the indicated neuronal markers. The following note duration: A and B, 1 week; C and D, 2 weeks; and E-H, 3 weeks. Arrows indicate long processes. MAP2 indicates microtubule-associated protein 2; DAPI, 4′,6′-diamidino-2-phenylindole. The color of the labels indicates location in the figure.

Rats not treated with doxycycline were allowed to survive for 2, 4, or 6 weeks. Transplanted HSP1 cells proliferated rapidly and formed large, circumscribed cell masses at 2 to 4 weeks both in the spinal cord (Figure 2C, D) and in the hippocampus (not shown). However, by 6 weeks, the cell masses in both the spinal cord and hippocampus had disappeared in 2 of 3 animals.

## EXTENT OF DIFFERENTIATION OF GRAFTED HSP1 CELLS

At low-dose or no doxycycline treatment, the grafted cells continued to divide, formed a cell mass, and showed Ki67-positive staining (**Figure 3**A). Their processes were short and not oriented parallel to the long axon tracts. The cells maintained their undifferentiated neuronal phenotypes as indicated by TUJ1 and nestin positivity (Figure 3C, E) and MAP2 negativity (Figure 3G).

At a 1-mg/mL doxycycline dose, transplanted cells stopped dividing, were Ki67-negative (Figure 3B), and extended long, thin processes, some more than 350 µm, that extended into the white matter and followed a trajectory similar to that of host axons (Figures 1C, D, 3F, H). These hM antigen–positive processes continued to express TUJ1 (Figure 3D) but also expressed nestin (Figure 3F). No hM antigen–positive processes or hN antigen–positive cells expressed MAP2. Thus HSP1 cells were incompletely differentiated at 4 weeks after transplantation. It is not known whether with longer survival, the cells could have become mature motor neurons.

## CAUSES OF HSP1 CELL LOSS

By 4 weeks, many HSP1 cells (but not host cells) showed fragmentation of their nuclei, suggesting that they were undergoing apoptosis (**Figure 4**A, B). This was confirmed by caspase immunohistochemistry. In an animal not treated with doxycycline and killed 4 weeks after transplantation, 11.9% of HSP1 cells were positive for activated caspase-3 (Figure 4C). An almost identical number (10.8%) were positive for activated caspase-9 (Figure 4D), suggesting that most of the dying cells were undergoing apoptosis via the cytochrome *c* pathway.

Interspersed among the apoptotic cells, but not in host tissue, many cells were ED1 positive (**Figure 5**A, B), a surface marker for activated microglia-macrophages. The cell mass was surrounded by large numbers of NK cells (Figure 5C, D), but these were relatively uncommon within the cell mass itself. Very few CD5-positive cells were observed (Figure 5E, F), suggesting that T cells in general were not a significant component of the immune response at the time examined.

## COMMENT

In a previous study,[1] HSP1 cells behaved like committed motor neuronal progenitors in vitro. The current study was originally undertaken in the hope that HSP1 might be developed as a potential therapy for spinal cord disorders that involve loss of motor neurons, for example, amyotrophic lateral sclerosis, postpolio syndrome, ventral root avulsion, and spinal cord injury. However, once transplanted into rat spinal cord, HSP1 cells showed a severe vulnerability to apoptosis, suggesting that they may be a model for xenotransplantation failure. Apoptosis has

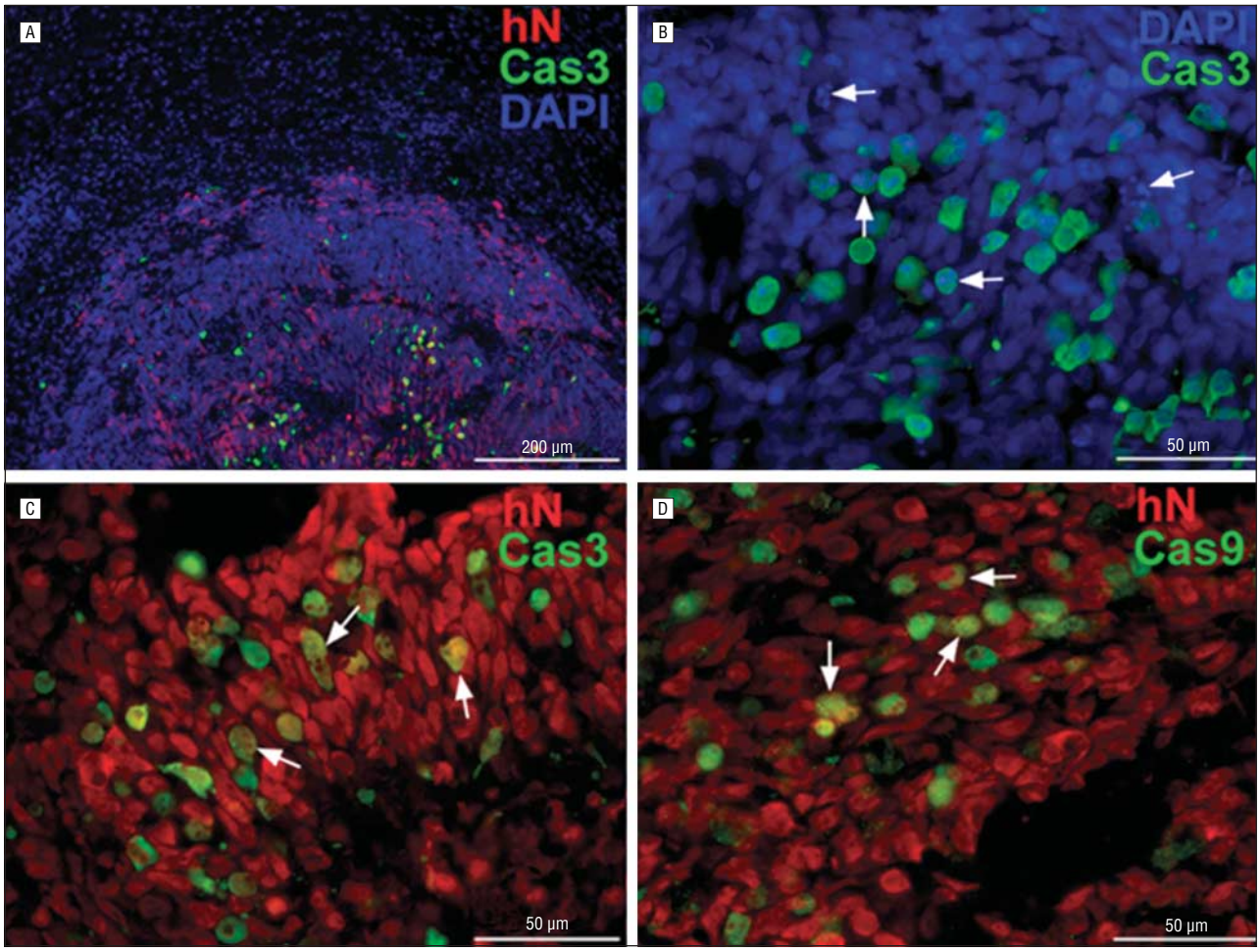


**Figure 4.** Apoptosis of transplanted HSP1 cells by caspase-9 (Cas9) pathway. A no doxycycline–treated animal survived 4 weeks and sections through the transplant in spinal cord were double labeled (arrows) for human nuclear (hN) antigen and caspase-3 (Cas3) (A-C) or caspase-9 pathway (D). Nuclear fragmentation (arrows) and caspase labeling were restricted to the transplant. DAPI indicates 4′,6′-diamidino-2-phenylindole. The color of the labels indicates location in the figure.

been seen in both allogeneic and xenogeneic dopaminergic neurons transplanted into parkinsonian animals.[5] It is not known whether different apoptotic pathways are involved.

## CAUSES OF HSP1 CELL DEATH

### Doxycycline Toxicity

We did not measure serum doxycycline levels, but animals took in at least 20 mg/d and cerebrospinal fluid levels would have reached at least 20 µg/mL. We have observed that at these conventional levels doxycycline was toxic to HSP1 cells in vitro. However, cells died even in non-doxycycline–treated animals. Although the cells disappeared more rapidly in doxycycline-treated animals, this could be owing to the in vivo proliferation of nonsuppressed cells before rejection took hold.

### Gliogenic Spinal Cord Environment

Adult neural stem cells in spinal cord generate only glia, although they can generate neurons in vitro or if transplanted into a neurogenic area of the brain, for example, hippocampus.[4] In one study, embryonic mouse stem cells generated both glia and neurons when transplanted into the injured rat spinal cord, but most surviving mature cells were either oligodendrocytes or astrocytes.[6] In other studies, pleuripotent rat embryonic stem cells generated only glia.[7] However, embryonic rat spinal cord NRP cells transplanted into uninjured adult rat spinal cord differentiated into neurons and not glia.[8] In the present study, HSP1 cells survived better in the hippocampus than in the spinal cord, but most cells had disappeared by 6 weeks, even in the no doxycycline–treated animals.

### Motor Neuron–Specific Programmed Cell Death

Motor neurons are especially difficult to transplant. For example, retroviral overexpression of human telomerase reverse transcriptase to immortalize progenitors from human fetal spinal cord, produced multiple neuron types, including motor neurons, in vitro.[9] When transplanted into the injured rat spinal cord, many cells differentiated into neurons and survived for 6 months, but most of the cells differentiated into GABAergic neurons. Graft-derived motor neurons were not described.[9] Even if grafted motor neurons survive, they fail to send axons into the

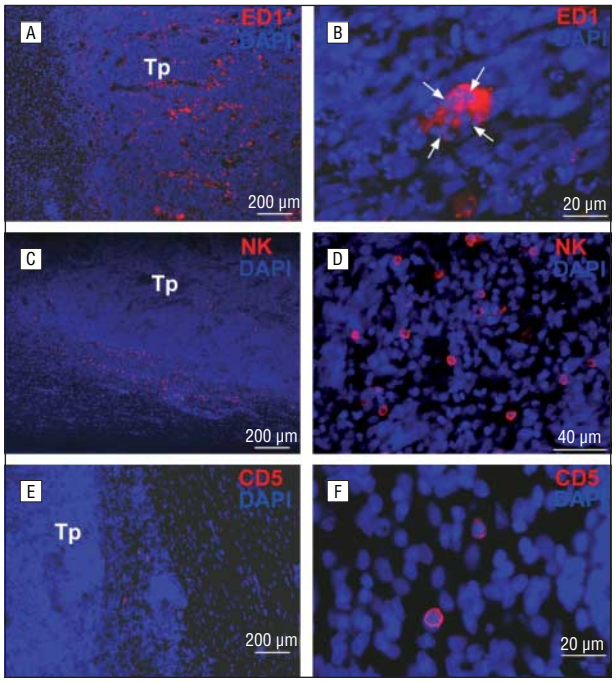


**Figure 5.** Host immunoresponse to HSP1 transplant. In the same animal as shown in Figure 4, sections were labeled for the indicated antigens. A and B, ED1-positive cells (activated microglia/macrophages) inside the transplant (Tp). Note ingested nuclear fragments (arrows). C and D, Natural killer (NK) cells surrounding the transplant. E and F, CD5-positive cells (T cells) were sparse. The color of the labels indicates location in the figure.

ventral roots, presumably because of myelin-associated growth inhibitory molecules in ventral white matter. Several experimental strategies have attempted to circumvent this problem by grafting neuronal progenitors into a peripheral nerve[10] or into the spinal cord and apposed to a peripheral nerve graft,[11] or by blocking intracellular signaling of myelin-associated growth inhibitors.[12] Signal factors that are important in specifying motor neurons during development may be useful in generating functional motor neurons from transplants of embryonic stem cells.[13]

During development, 50% of motor neuronal progenitors undergo programmed cell death after differentiation owing to competition by their axons for trophic factors released by muscle cells. The first motor neuron to innervate a myocyte captures its trophic factor supply and induces its down-regulation.[14] In chick embryos, this characterizes motoneuron loss between E6 and E10. However, at E4 to E5, motor neuron precursors undergo apoptosis independent of trophic factors and myocyte innervation.[15] This does not seem to apply to undifferentiated HSP1 cells, which are expandable indefinitely.

## IMMUNOLOGIC REJECTION

The mechanisms of immunologic rejection of neural xenografts in the central nervous system (CNS) have not been extensively studied.[16] In nonneural tissues, after the hyperacute phase, graft rejection involves T-cell–mediated immunological attack directed against MHC or other antigens expressed on the surfaces of cells.[2] Porcine dopaminergic neuronal grafts xenotransplanted into mice survived longer in animals lacking NK-T cells but not in recipient mice lacking NK cells.[17] Neurons in general, and neuronal progenitors in particular, express lower levels of MHC than other cell types and it has been suggested that the relative immunological privilege of the CNS is due in part to the intrinsic properties of neurons.[18] However, motor neurons and nigral dopaminergic neurons express MHC class I heavy chain and $\beta_2$-microglobulin messenger RNAs, and it has been speculated that this may be related to the selective vulnerability of these neurons in neurodegenerative diseases.[19] These neurons also express interferon-$\gamma$ receptor, which when activated, causes up-regulation of MHC antigens. Whether HSP1 cells express MHC antigens is still not known. However, the scarcity of CD-5–labeled cells in the vicinity of the transplanted cells in the present study suggests that T-cell–mediated immunological attack may not play a role in the apoptotic cell death of HSP1 cells. What was striking, however, was the large number of NK cells surrounding the HSP1 cell mass. The role of NK cells in xenograft rejection in the CNS is unknown, although NK cell–dependent cytotoxicity has been implicated in the mechanism of kidney xenograft[20] but not allograft rejection.

## CONCLUSION

It is ironic that in a part of the body that is commonly considered "immunologically privileged," xenotransplantation should be so difficult. In the present study, immortalized human motor neuronal progenitors transplanted into rat spinal cords died rapidly by apoptosis via the cytochrome *c*/caspase-9/caspase-3 pathway. The scarcity of T cells in the region of the transplanted cell mass suggests that the cell death may not be T cell mediated. Although doxycycline toxicity may have contributed to cell death, and the cytochrome *c*/caspase-9 pathway is often involved in neurotoxic apoptosis, in the present study, cells died rapidly even without doxycycline. Nor was the gliogenic spinal cord environment an adequate explanation, since rapid cell death was also seen in the hippocampus. Our results suggest that the xenotransplantation failed because of apoptotic cell death, possibly involving NK cells.

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